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14. ABSTRACT

This DoD Innovation grant was based on the hypothesis that transmissible ER stress (TERS) promotes the Epithelial to Mesenchymal Transition (EMT) in differentiated prostate cancer cells, programming cancer towards a different phenotype and greater invasive characteristics. The hypothesis predicted a new and potentially important mechanism in tumorigenesis. Through the work performed during the last year, we have been able to demonstrate a link between prostate tumor ER stress, EMT, and enhanced invasiveness. The study is not finished yet but we can confidently say that the premises of the original hypothesis have been experimentally validated.

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Annual Report

DOD Award: W81XWH-12-1-0156
Principal Investigator: Maurizio Zanetti, M.D.

Abstract

This DoD Innovation grant was based on the hypothesis that transmissible ER stress (TERS) promotes the Epithelial to Mesenchymal Transition (EMT) in differentiated prostate cancer cells, programming cancer towards a different phenotype and greater invasive characteristics. The hypothesis predicted a new and potentially important mechanism in tumorigenesis. Through the work performed during the last year, we have been able to demonstrate a link between prostate tumor ER stress, EMT, and enhanced invasiveness. The study is not finished yet but we can confidently say that the premises of the original hypothesis have been experimentally validated.

Introduction

How prostate tumor cells cope with tumor microenvironmental stress often dictates successful tumor outgrowth. This may entail the co-opting of a variety of processes, including resistance to programmed cell death or increased vascularization. While these processes enable successful primary tumor growth, they do very little to explain metastasis, the often fatal feature of cancer. One possibility to explain how epithelial tumor cells acquire motile characteristics is through a process known as the epithelial to mesenchymal transition (EMT). This model posits that transformed epithelial cells become polarized and undergo epigenetic changes towards a mesenchymal phenotype, allowing for greater motility and ultimately escape from the primary tumor. While much emphasis has been placed on probing the genetic machinery of this intriguing cellular process, there has been very little consideration for what tumor or tumor microenvironmental cues may initiate EMT.

Recent evidence suggests that prostate tumor cells have an enhanced unfolded protein response (UPR), a programmed cellular response tumor cells employ to cope with endoplasmic reticulum (ER) stress. Indeed, recent reports indicate the master protein of the UPR, Grp78, is a necessary component for prostate cancer tumorigenesis. UPR activation is often elicited by *noxae* in the tumor microenvironment. Reports show that the UPR enables tumor cell survival, promotes chemoresistance, and represents a possible link to the initiation of EMT. The induction of UPR in tumor cells also drives the secretion of Lipocalin 2 (LCN2) and interleukin (IL)-6, cytokines that can promote EMT in several epithelial cancers.

We recently reported that ER-stressed prostate tumor cells influence tumor-associated myeloid cells in a cell-extrinsic manner, polarizing them into an inflammatory phenotype, an event termed transmissible ER stress (TERS). Provided this novel extrinsic role of the tumor UPR and the potential role the UPR has in EMT, the tumor UPR, and more specifically TERS, may represent the cues tumor cells receive to undergo EMT and increased invasiveness.

Summary of Experimental Data

Under the aegis of this DoD grant, we have begun testing the hypothesis that endoplasmic reticulum (ER) stress in prostate cancer cells direct EMT changes in otherwise unstressed cancer cells through cell extrinsic effects. To this end, we stressed human PC3 prostate cancer cells with a canonical ER stress drug, thapsigargin, or its vehicle control, to generate ER stress or vehicle (Veh) conditioned

media. Because the former can transmit a ER stress response in receiver cells, its effect has been termed transmissible ER stress (TERS).

We sought to determine if prostate cancer cells cultured over five days in TERS (which was re-supplemented daily) undergo a UPR, as indicated by the fold upregulation of the master UPR gene, *Grp78* (Fig 1a).

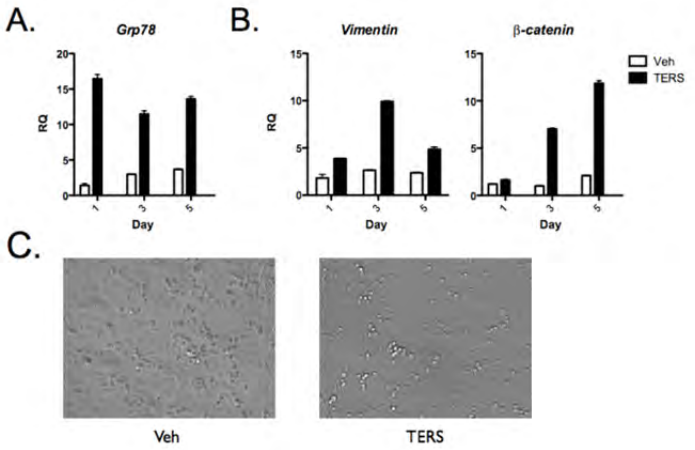

Figure 1: TERS treated prostate cancer cells undergo a UPR and EMT.
PC3 cells were treated to TERS for 5 days and probed for a A. UPR via *Grp78* and B. EMT, as determined by *vimentin* and *ß-catenin*, respectively. C. Bright-field visualization of treated cells after 72 hrs.

After establishing that the gene is highly upregulated following exposure to TERS, we sought to determine if prostate cancer cells also undergo EMT as determined by RT-qPCR analysis of EMT-associated genes (*ß-catenin* and *Vimentin*) (Fig 1b). TERS treated PC3 cells in fact upregulated both genes for all five days. Moreover, TERS treated cells display a more mesenchymal phenotype than vehicle treated cells, which display a traditional epithelial phenotype (Fig 1c).

Having established that TERS drives EMT polarization, we probed other known facilitators of EMT. To our surprise, we found no significant change during TERS treatment in the gap junction protein E-cadherin at both the genetic and protein levels (Fig 2a,b). In contrast the genes for *Il-6* and lipocalin-2 (*Lcn2*), cytokines that can facilitate EMT in various tissues, are greatly upregulated during times of TERS treatment (Fig 2c).

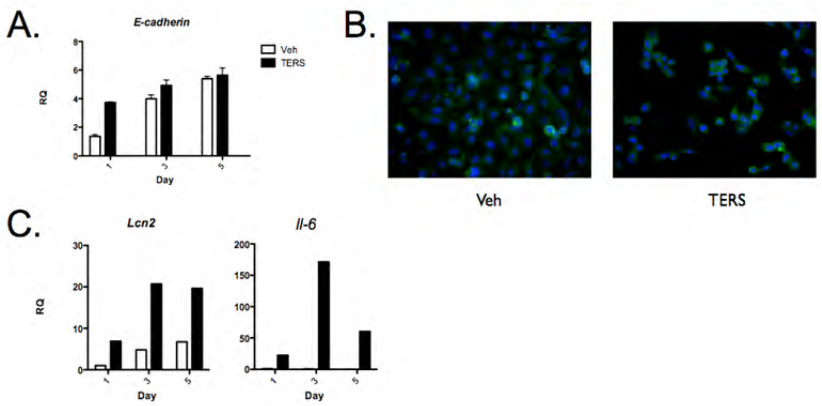

Figure 2: TERS evoked EMT does not affect E-cadherin but activates EMT cytokines. A. E-cadherin expression of TERS treated PC3 for five days. B. Immunostain for E-cadherin (green) and nuclear (blue) after three days of TERS stress. C. Genes expression for known EMT cytokines lipocalin 2 (Lcn2) and Il-6

Collectively, these results suggest that TERS treated prostate cancer cells undergo a partial EMT polarization, even after a 24 hours exposure to TERS.

We further interrogated Twist, a recently proposed EMT facilitator, and probed whether its induction can be mediated by TERS. Two new reports suggest Twist is necessarily upregulated during EMT

enabling the extravasation of cancer cells from a primary tumor to occur. Further, Twist necessarily is downregulated once cancer cells have colonized distal metastasis.

PC3 cancer cells were treated to TERS for three days followed by a refractory period of five days in which standard culture media was used (Fig 3a). Over this period of time, cells were harvested daily and probed for their expression of *Twist1* (Fig 3b). Our findings show that TERS is a potent inducer / maintainer of Twist, and once removed from culture conditions, prostate cancer cells can be restored to their original epithelial state.

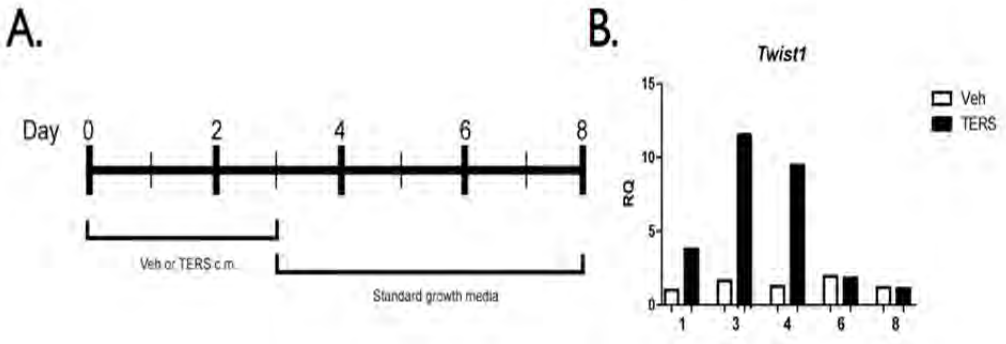

Figure 3: Twist expression is dependent of TERS.
A. Dosing regimen of TERS over PC3 over eight days.
B. *Twist* gene expression over treatment periods

Emerging data suggest that TERS treated PC3 cells do not have enhanced motility, as determined by trans-well and wound healing assays. However, these results are provisional and need to be repeated.

We have also explored the role of TERS on telomerase reverse transcriptase (*Tert*) expression. *Tert* upregulation is a common hallmark of cancer cells as it allows for lengthening of the telomeres and the potential for an unlimited number of cell divisions. ß-catenin has been shown to bind the *Tert* promoter and increase *Tert* expression levels. Since ß-catenin is upregulated when cells are exposed to TERS, we hypothesized that TERT levels may also be upregulated. To test this hypothesis, PC3 cells were grown on glass slides and treated with Veh or TERS conditioned media. The resulting cells were then stained for TERT and DAPI. The slides were imaged using fluorescent confocal microscopy (Figure 4).

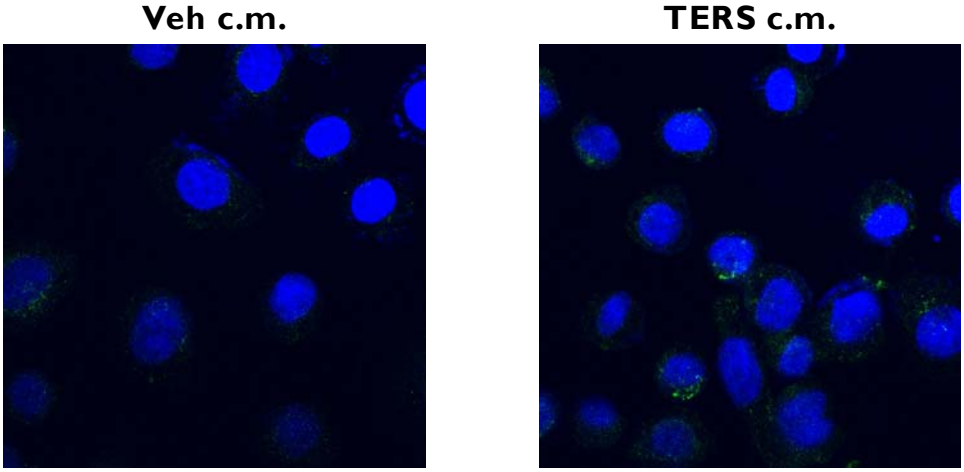

Figure 4: TERS induces TERT expression
PC3 cells were grown on glass slides and treated with Veh or TERS conditioned medium (c.m.) for 48h. Cells were then fixed and stained for DAPI (blue) and anti-human TERT (green). Cells were then mounted and visualized using fluorescence confocal microscopy.

Figure 4 shows that cells exposed to TERS c.m. have significantly greater expression of TERT in the cytoplasm compared to cells treated with Veh c.m. We currently are repeating this results and will also probe the molecular upregulation of the gene as well.

Finally, we sought to uncover a functional role TERS may play in prostate cancer cells. Previous experiments demonstrate that TERS does not promote migration or metastatic ability in cancer cells despite their upregulation in many EMT markers. An idea was then fostered that perhaps TERS may play a role in the survival of cancer cells instead of their migration. To test this hypothesis, PC3 cells were treated with either Veh or TERS c.m. for 48 hours. The conditioned media was then washed off and the cells were placed under normal growth media for 24 hours. Veh or TERS "primed" cells were then plated and treated with thapsigargin at various concentrations for 48 hours. Cells were harvested for the stress levels as determined by expression for Grp78 (Fig 5a). TERS primed cells markedly reduce their stress response compared to vehicle primed cells during times of ER stress. We sought to quantify whether this reduction in stress protects cells from apoptosis. Using crystal violet staining, we find that TERS primed cells are healthier than vehicle primed cells, suggestive that TERS priming may protect tumor cells during times of various ER stress.

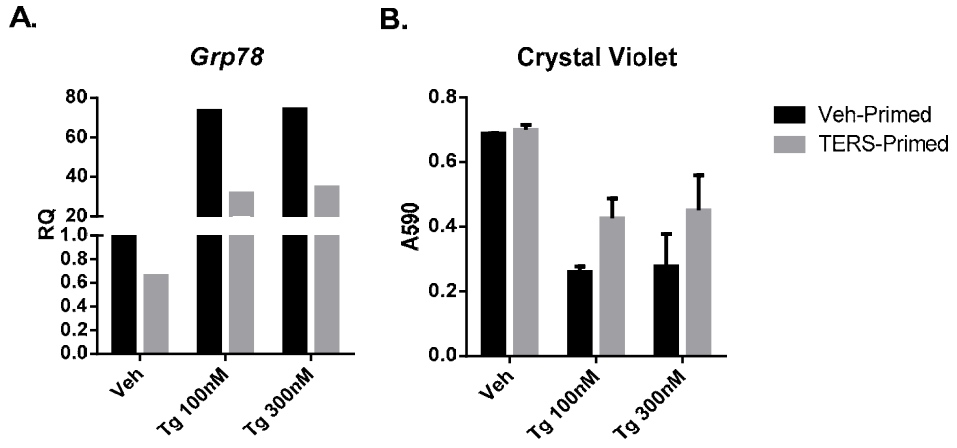

Figure 5: TERS-primed cells have a decreased UPR response and greater plate attachment in the presence of thapsigargin.
PC3 cells were treated for 48h with thapsigargin at 100nM and 300nM concentrations following priming with Veh or TERS c.m. A. The expression levels for *Grp78* were quantified by RT-qPCR. B. Treated cells were fixed and stained with crystal violet. Cellular attachment was quantified using an absorbance reader at A_{590}.

The results from Figure 5 demonstrate that PC3 cells that have been previously exposed to TERS now have a significantly reduced expression of *Grp78* compared to cells without exposure. In the presence of a stressor (Thapsigargin), TERS-primed cells have a lower UPR response. This suggests that TERS may condition prostate cancer cells to handle and better survive a secondary stress.

We sought to further elucidate if in fact this ressitance to secondary UPR induction ("fitness") plays a role in more physiologically-relevant stressful conditions. To this end, we cultured PC3 cells pre-treated with TERS or vehicle (48 hours on, 24 hours off) in glucose free, serum free conditions. After 48 hours, cells were harvested and probed for their UPR by measuring Grp78 expression (Fig 6a). TERS primed PC3 cells grown in nutrient deprived conditions had a reduced UPR than those of their veh primed controls.

To assess if such a reduced UPR may lend TERS primed cells to a more fit status, we assessed the viability of these cultures after 72 hours using Annexin V staining to assess apoptosis (Fig 6b). While normal growth conditions yield markedly similar distributions of apoptotic and viable cells, nutrient deprived conditions had markedly different effects in vehicle primed versus TERS primed cells. Of note, there exists a 45% viable population in TERS primed cultures while only 3% in vehicle primed cultures. Our results indicate, therefore, that TERS primed cells are much more fit to survive stress than their vehicle counterpart.

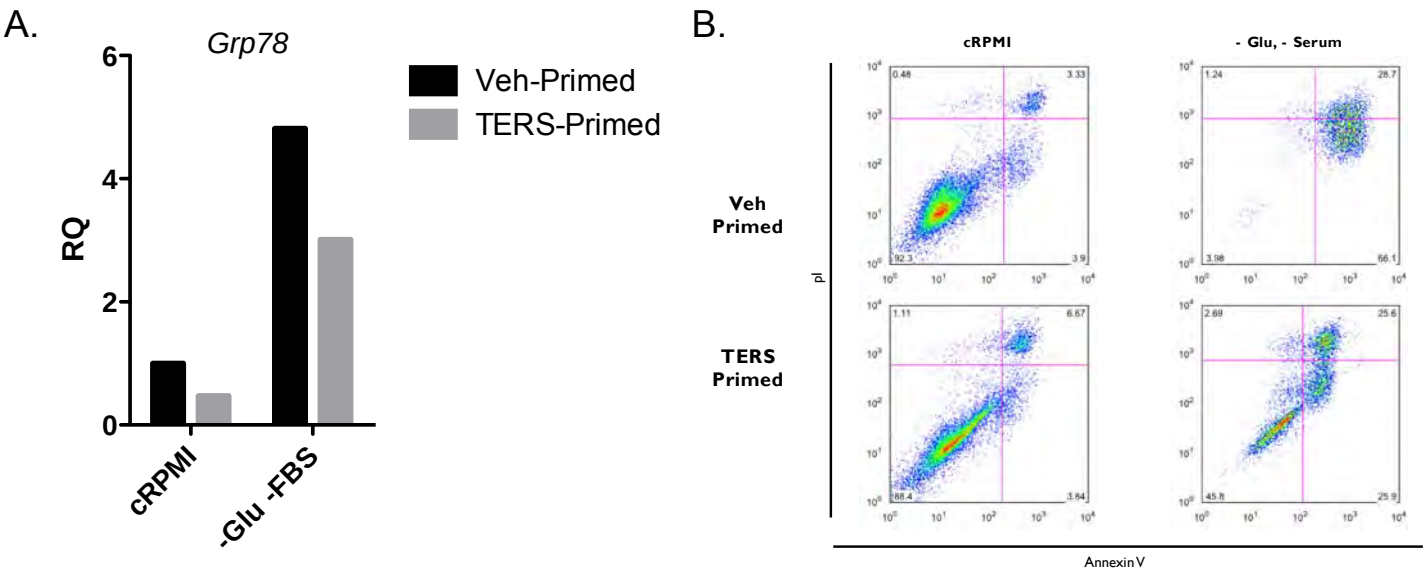

Figure 6: TERS-primed cells have a decreased UPR response and increased survival during times of nutrient deprivation.
PC3 TERS or vehicle primed cells were grown in standard growth media (cRPMI) or glucose and serum free growth media (-Glu,-FBS) for 48h. A. The expression levels for *Grp78* were quantified by RT-qPCR. B. Treated cells were analyzed for apoptosis by probing for annexin V positivity.

To further appreciate a fitness advantage TERS primed cells have against various cellular insults, we asked whether these cells would too be protected from chemotherapy induced cytotoxicity. We elected to use bortezomib (Velcade), a proteasome inhibitor that has emerged as a candidate molecule in a variety of studies the past several years to be used as an adjuvant and neoadjuvant in prostate cancer. Following 48 hours of TERS priming and a 24 hour unstressed period, PC3 cells were treated to bortezomib at increasing concentrations. Cells were then harvested after 24 hours of bortezomib treatment and assayed from their stress response and apoptotic status.

Bortezomib stress of TERS primed and vehicle primed cells yields similar results to that of nutrient deprivation. TERS primed PC3 cells undergo a reduced UPR, as determined by Grp78 and Chop, to that of their vehicle primed cohort (Fig 7a). More importantly, TERS primed cells survive markedly better in times of bortezomib treatment to that of vehicle primed cells ranging from low levels of bortezomib (10 nM) large doses (1 μM) (Fig 7b). Collectively, these results suggest TERS priming may play an insidious role in protecting tumor cells from a variety of noxae, both tumor borne and therapeutic derived.

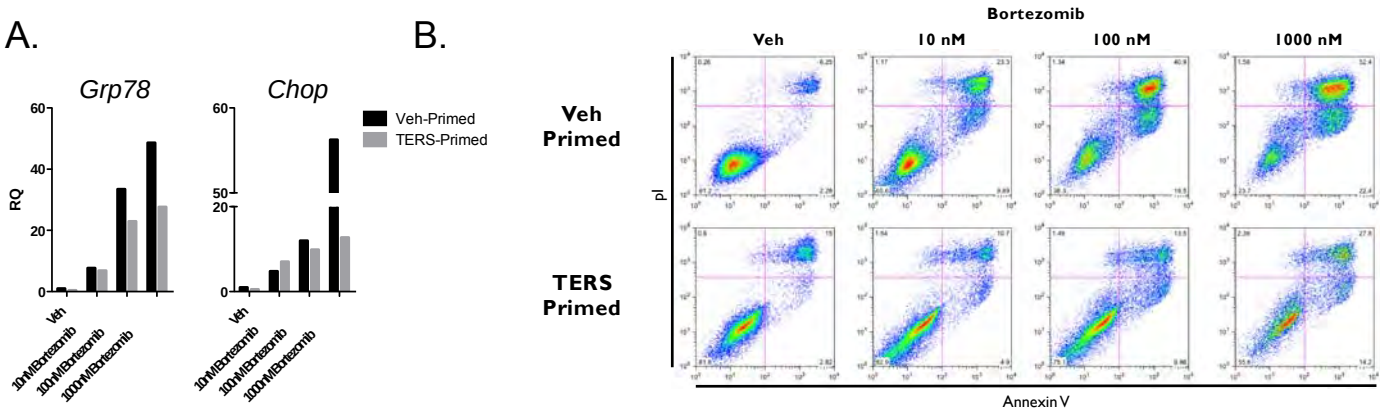

Figure 7: TERS-primed cells are protected from bortezomib induced stress and killing.
PC3 TERS or vehicle primed cells were treated for 24h with bortezomib at 10, 100, and 1000 nM. A. The expression levels for *Grp78* and *Chop* were quantified by RT-qPCR. B. Treated cells were analyzed for apoptosis by probing for annexin V positivity.

Overall, the results we are pleased to report here reveal an unappreciated role of the cell extrinsic role a prostate tumor UPR may play. Though these results are compelling, larger questions still persist including the durability of the TERS primed phenotype as well as if TERS primed tumor cells have increased invasiveness in vivo. We are currently pursuing and intend to answer these questions in the coming months.

Key Research Accomplishments

- Prostate cancer cell lines, when undergoing a UPR through transmissible ER Stress (TERS), exhibit many key characteristics of an epithelial-to-mesenchymal transition.
- TERS-treated prostate cancer cells also undergo changes that are not typical of an EMT such as an upregulation in E-cadherin
- TERS-treated cells undergo a change in morphology similar to those seen undergoing EMT
- Various EMT cytokine genes, including IL-6 and Lipocalin 2, are elevated in TERS treated prostate cancer cells
- TERS-treated cells exhibit an activation of the Wnt-signaling pathway
- Twist expression is directly linked with the presence of TERS
- TERS induces TERT expression
- Prostate cancer cells exposed to TERS are more fit than those who haven't to handle subsequent exposure to stress inducing conditions.

Reportable Outcomes

We have verified the basic tenet of the DoD application that transmissible ER stress is causative of changes in unstressed prostate cancer cells that are consistent with EMT except for a down-regulation of E-cadherin. While we are work towards understanding the significance of this partial

EMT, we have elevated our findings to report that these primed cells have markedly increased fitness to vehicle primed cells during times of various cellular stress.

Conclusion

The work to date allows us to say that transmissible ER stress is causative of changes in unstressed prostate cancer cells that are consistent with EMT except for a down-regulation of E-cadherin. Experiments that will performed in future months through a NCE will bring to conclusion this study and we will begin writing a manuscript for publication based on the new findings discussed herein.

References

No paper has been submitted to publication yet but it will as soon as the research that has been outlined above reaches a point that a paper can be safely be prepared and submitted to a peer-reviewed journal.